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## Purification and Biologic Characterization of Plasma-Derived Megakaryocyte Growth and Development Factor

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The isolation and cloning of the ligand for the cytokine receptor, Mpl, have been recently described. In this report we present details of the purification of this novel cytokine (megakaryocyte growth and development factor [MGDF]) from aplastic canine plasma. Two forms of canine MGDF, with apparent molecular weights of 25 kD and 31 kD and sharing a common N-terminal amino acid sequence, were isolated. The sole contaminant detected in purified 25-kD or 31-kD MGDF was canine Ig. Canine MGDF is characterized as a human megakaryocyte colony-stimulating factor that acts synergistically with human recombinant stem cell

factor but not interleukin-3. MGDF also appears to be physiologically regulated in response to platelet demand. In canines and murine models, serum levels of MGDF activity peak during the thrombocytopenic periods after irradiation, 5-fluorouracil, or antiplatelet antisera injections. These data indicate that the megakaryocyte-stimulating activity that accumulates in plasma in response to platelet losses is a novel cytokine that functions through an interaction with the Mpl cytokine receptor.

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**R**ECENTLY, A LIGAND for the cytokine receptor Mpl<sup>1,2</sup> has been isolated and/or cloned by several independent groups.<sup>3</sup> This novel cytokine has been named Mpl ligand (ML),<sup>4</sup> thrombopoietin (TPO),<sup>5-7</sup> megapoietin,<sup>8</sup> or megakaryocyte growth and development factor (MGDF).<sup>9</sup> The recombinant ligand acts as a megakaryocyte colony-stimulating factor (Meg-CSF) on murine<sup>6</sup> or human<sup>7</sup> bone marrow cells and as a platelet-stimulating factor in murine *in vivo* studies.<sup>4-6</sup> It also induces platelet glycoprotein expression within human bone marrow populations<sup>4</sup> and stimulates the generation of megakaryocytes from human<sup>9</sup> or murine<sup>10</sup> hematopoietic progenitor cells.

In this report, the details of the purification of this new factor from aplastic canine sera, an acknowledged source of human Meg-CSF,<sup>11-14</sup> are presented. Effects of purified canine MGDF on human, *in vitro* megakaryopoiesis are presented, as are data relating serum MGDF levels to platelet counts. These studies indicate that most, if not all, of the megakaryopoietic activity induced by thrombocytopenia and/or marrow aplasia<sup>11-13,15-21</sup> can be attributed to this new cytokine.

### MATERIALS AND METHODS

#### Human Subjects and Plasma Products

Heparinized platelet-poor human AB plasma was obtained from healthy adult volunteers with informed consent. Leukapheresis units were purchased from HemaCare (Sherman Oaks, CA). Heparinized, aplastic canine plasma was produced as described previously<sup>11</sup> except that the animals received a total of 450 rad  $\gamma$  irradiation from a Co<sup>60</sup> source. Plasma was collected 10 to 14 days after irradiation.

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#### Production and Purification of Mpl-X

The recombinant, extracellular domain of the murine *mpl* gene product (Mpl-X)<sup>9</sup> was purified from CHO cell culture supernatant as follows. Supernatant (100 L) was concentrated approximately 10-fold by ultrafiltration (30,000 molecular weight cut off; S10Y30, Amicon, Danvers, MA) and diafiltered against 10 mmol/L Tris-HCl, pH 8.5. The concentrate was applied to a column of Q-Sepharose Fast Flow (14 × 11 cm; Pharmacia, Piscataway, NJ). The column was washed with 10 mmol/L Tris-HCl, pH 8.5, and eluted at 75 mL/min with an 85 L linear gradient to 0.3 mol/L NaCl in the same buffer. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Mpl-X was detected using antisera generated against previously purified Mpl-X.<sup>9</sup> Positive fractions, corresponding to approximately 150 mmol/L NaCl, were pooled, diafiltered against 10 mmol/L sodium phosphate, 0.01 mmol/L CaCl<sub>2</sub>, pH 6.8, and applied to an hydroxylapatite column (7 × 5.2 cm; HA-Ultrogel; Sepracor, Marlborough, MA) at a flow rate of 13 mL/min. The unbound fraction, containing purified Mpl-X, was diluted into phosphate-buffered saline (PBS) and stored at -80°C.

#### MGDF Assays

An assay using a murine cell line in which the murine Mpl gene was expressed (32D/Mpl+) has been previously described.<sup>9</sup> The assay was performed by serially diluting test samples (1:1 for 10 dilutions) into wells of Terasaki-style microtiter plates (Vangard, Neptune, NJ) at a total volume of 15  $\mu$ L. One unit of activity is defined as the amount of material that permits survival of 200 cells per well from an initial inoculum of 1,000 cells per well after 48 hours of culture. This assay had a logarithmic dose response relationship ( $r^2 = .95$ ) from 200 to 100,000 U/mL and 200 U/mL was the limit of detection. The starting material averaged  $4,400 \pm 539$  U/mL.

An assay using megakaryocyte induction from cultures of CD34<sup>+</sup> peripheral blood cells has been previously described.<sup>9,14</sup> In this assay, 5,000 CD34<sup>+</sup> peripheral blood cells were stimulated with MGDF, and the number of megakaryocytes resulting after 8 days of culture was enumerated. This assay was responsive to 2% to 20% irradiated canine plasma. The response to purified MGDF was linear from 250 to 2,500 U/mL ( $r^2 = .98$ ), and the limit of detection was 100 U/mL. The responses to MGDF in both assays were completely inhibitable with 5 to 10  $\mu$ g/mL Mpl-X.

#### Purification of MGDF

Unless noted otherwise, all operations were performed at 4°C. Frozen aplastic canine plasma (20 L) was thawed overnight and

centrifuged at 11,000g for 6 hours. The plasma was diluted twofold in PBS, pH 7.3, containing 0.01% sodium azide (PBS/azide) and passed through a 0.2  $\mu$ m filter; 500 cm<sup>2</sup> Serum Capsule; Gelman). The clarified plasma was applied to a column of immobilized wheat germ agglutinin (1 L; 10  $\times$  12 cm; E-Y Laboratories) equilibrated in PBS/azide at a flow rate of 35 mL/min. The column was washed with PBS/azide and then with 0.5 mol/L NaCl in 20 mmol/L Tris-HCl, pH 8. Bound material was eluted (0.35 mol/L GlcNAc, 0.5 mol/L NaCl, 20 mmol/L Tris-HCl, pH 8), concentrated (10,000 molecular weight cut off; YM-10; Amicon), adjusted to 0.2 mol/L NaCl by dilution, and applied to a 20 mL Mpl-X/CNBr activated Sepharose 6B (Pharmacia) column (2.6  $\times$  4.2 cm, 1.5 mg murine Mpl-X/mL of column) at 0.9 mL/min. The column was washed with 40 mL of PBS/azide at 1.5 mL/min, followed by 405 mL of 10 mmol/L Tris-HCl, 0.5 mol/L NaCl, 1 mmol/L CHAPS, pH 8.0. The column was eluted with 20 mmol/L CAPS, 1 mol/L NaCl, 5 mmol/L CHAPS, pH 10.5. Fractions were neutralized immediately on collection.

Mpl-X affinity-purified MGDF was concentrated and desalting using 20 mmol/L Tris-HCl, 5 mmol/L CHAPS, pH 8.7, by ultrafiltration (YM-10; Amicon) to a final volume of 58.5 mL. The pool was loaded at 0.5 mL/min onto a Mono Q HR 5/5 (Pharmacia) column equilibrated in the same buffer. The column was eluted with a linear gradient to 0.36 mol/L NaCl in 20 mmol/L Tris-HCl, 5 mmol/L CHAPS, pH 8.7, over 27 minutes. The column was washed with a 6-minute gradient to 0.54 mol/L NaCl and finally with a step wash at 0.9 mol/L NaCl. One-milliliter fractions were collected. Peak (see Results) was concentrated (Centricon-10; Amicon), brought to 50% SDS, and injected onto a Superdex 200 HR 10/30 (Pharmacia) column equilibrated in 50 mmol/L Tris-HCl, 0.1% SDS, pH 7.5, at a flow rate of 0.3 mL/min at room temperature. Fractions of 0.3 mL were collected. Superdex 200 fractions containing MGDF activity were pooled, concentrated (Microcon-10; Amicon), and applied to a 100 mm C4 reverse-phase column (SynChropak RP-4). The column was equilibrated in 0.04% TFA in water (A buffer); B buffer was 0.035% TFA in 80% acetonitrile. After injection of the sample, a linear gradient to 45% B over 4 minutes was performed, followed by a linear gradient to 75% B over 40 minutes. The flow rate was 0.3 mL/min. Amino acid sequence determination of proteins within selected fractions was performed as described.<sup>9</sup>

Electrophoresis and gel elution experiments were performed as previously described.<sup>9</sup>

#### Megakaryocyte Colony-Forming Unit (CFU-Meg) Assay

CD34<sup>+</sup> cells were isolated from mononuclear cells from leukapheresis units<sup>14</sup> with a CD34-isolation kit (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. CD34 purity was always greater than 94% as determined by immunostaining with HPCA-1 and HPCA-2.<sup>14</sup> Cells were plated at 20,000/mL in 15% irradiated, platelet-poor, human AB plasma in Iscove's modified Dulbecco's medium (IMDM) with additives as described by Mazur et al.<sup>12</sup> In some experiments, human recombinant interleukin-3 (IL-3) or stem cell factor (SCF) were included (Amgen, Inc., Thousand Oaks, CA). Triplicate wells of 300  $\mu$ L/well (6,600 cells) were incubated for 12 days at 37°C, 5% CO<sub>2</sub> in air. Plates were fixed with methanol:acetone and stained with antibodies to GPIb and GPIIb/IIIa (Biodesign, Kennebunkport, ME) and a secondary goat-antimouse fluorescein isothiocyanate (FITC; Southern Biotechnology Associates, Inc., Birmingham, AL). This procedure was followed by nuclear staining with 2.5  $\mu$ g/mL solution of propidium iodide (Sigma, St. Louis, MO) in 0.1% citrate. Colonies were defined as discrete groupings of three or more brightly fluorescent cells by inverted fluorescent microscopy (Olympus, Lake Success, NY) at 100 $\times$  magnification. In cases of excessive growth, confluent fields were counted as 3 colonies.

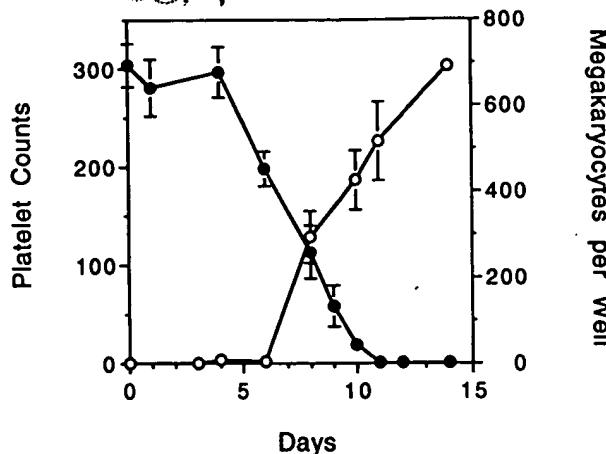


Fig 1. Platelet counts and megakaryocyte stimulating activity in canine plasma postirradiation. Animals were irradiated with 450 rad at day 0. Platelet counts ( $\times 1,000/\mu\text{L}$ ) were determined on the indicated days (●). Heparinized plasma was collected as indicated and used at 10% on CD34<sup>+</sup> cells. Megakaryocytes per well were enumerated after 8 days (○). (n = 9 animals except for day 14, where n = 1.)

#### Murine Models of Thrombocytopenia

Adult DBA and Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). 5-Fluorouracil (5-FU) was administered to DBA mice in a single intraperitoneal injection at 150 mg/kg body weight. Balb/c mice were  $\gamma$ -irradiated with a single dose of 500 rad (GammaCell 400; Nordion International, Ontario, Canada). Rabbit antimouse platelet antisera (APS)<sup>23</sup> or normal rabbit sera (NRS) was administered to DBA mice in a single 100- $\mu$ L intraperitoneal injection of sera diluted 1:5. Platelet counts were determined in a Sysmex blood cell analyzer (TOA Medical Electronics, Kobe, Japan) as described.<sup>24</sup> Sera were collected and assayed for MGDF activity in the 32D/MPL+ assay.

#### Data Analysis

Results are presented as mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using the two-tailed Student's *t*-test.

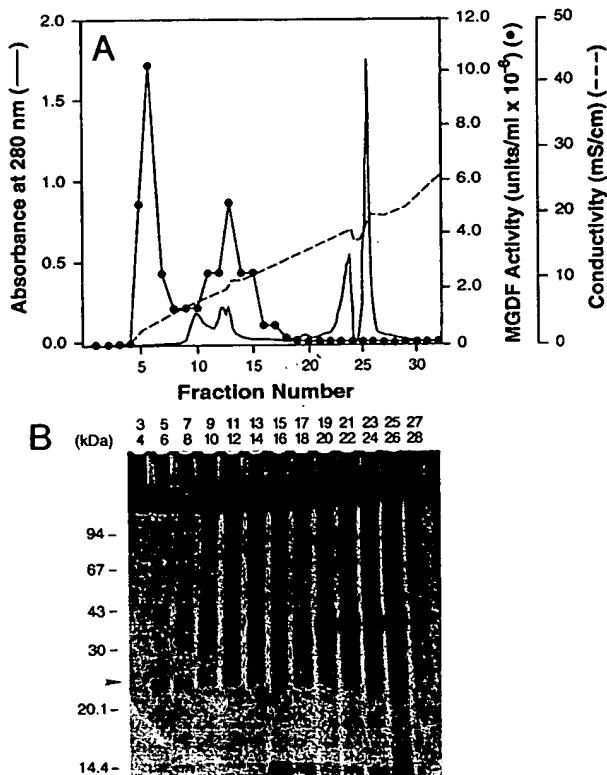
#### RESULTS

##### Purification of Canine MGDF

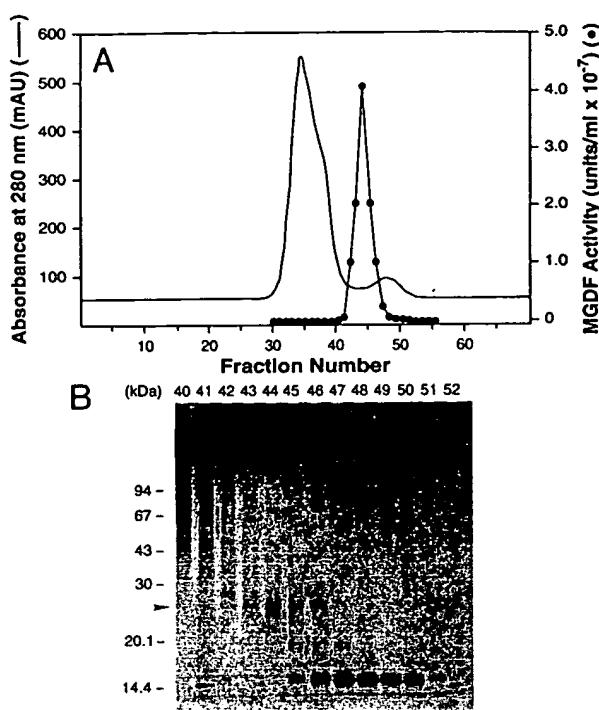
Irradiation of animals results in aplasia and thrombocytopenia accompanied by the appearance of megakaryopoietic activities in the serum or plasma.<sup>7,11,15,17,25</sup> Plasma from irradiated animals was used to develop a liquid culture system in which human megakaryocytes developed from CD34<sup>+</sup> progenitors.<sup>14</sup> This assay system was subsequently used to monitor the appearance of MGDF in relationship to platelet levels in irradiated dogs (Fig 1). A sharp increase in plasma activity could be observed 8 days after irradiation at a time when the platelet count had decreased to 30% of normal. Plasma MGDF activity continued to increase as the platelet counts decreased to their nadir of 1,000 to 5,000/ $\mu$ L. Equivalent results were seen using either serum or plasma collected after irradiation.

The purification of MGDF from canine plasma, collected

10 to 14 days postirradiation, was achieved by a combination of affinity (wheat germ agglutinin and Mpl-X receptor) chromatography, ion exchange, gel filtration, and reverse-phase high performance liquid chromatography (RP-HPLC). No MGDF activity was detected in the unbound fraction of either affinity column. The resulting pool was loaded onto a Mono-Q ion exchange column. The elution profile of this column showed the presence of two major peaks of activity (Fig 2A), and SDS-PAGE analysis indicated a correlation between MGDF activity and the presence of a 25-kD protein visible in silver-stained gels (Fig 2B). Elution experiments (data not shown) from SDS gels using aliquots of Mono-Q ion exchange peaks 1 and 2 showed that most of the MGDF activity comigrated with the 25-kD band, although some activity was also eluted from the 30- to 34-kD region of the gels, indicating at least two forms of MGDF activity. Based on these results, fractions corresponding to peak 1 were combined and separated by SDS-PAGE, and the 25-kD band (approximately 1  $\mu$ g by Coomassie staining) was cut from the gel. An in-gel proteolytic digestion failed to release sufficient amounts of peptide for sequence determination. Further purification efforts used peak 2.



**Fig 2.** Anion-exchange chromatography of MGDF. Active fractions from Mpl-X affinity columns were pooled and loaded onto a Mono Q HRS/5 column as described in Materials and Methods. (A) A representative profile is shown. Peak 1, fractions 5 to 8. Peak 2, fractions 9 to 15. MGDF levels were determined with the 32D/Mpl<sup>+</sup> cell line. (B) Aliquots of fractions were analyzed on 14% SDS-PAGE under nonreducing condition and silver-stained. Arrow indicates position of 25-kD MGDF.



**Fig 3.** Gel filtration chromatography of MGDF. MGDF peak 2 from the Mono Q fractionation was injected onto a Superdex 200 HR10/30 column as described in Materials and Methods. (A) A representative profile is shown. MGDF levels were determined with the 32D/Mpl<sup>+</sup> cell line. (B) Aliquots of fractions were analyzed by 14% SDS-PAGE under nonreducing conditions and silver stained. Arrow indicates position of 25-kD MGDF.

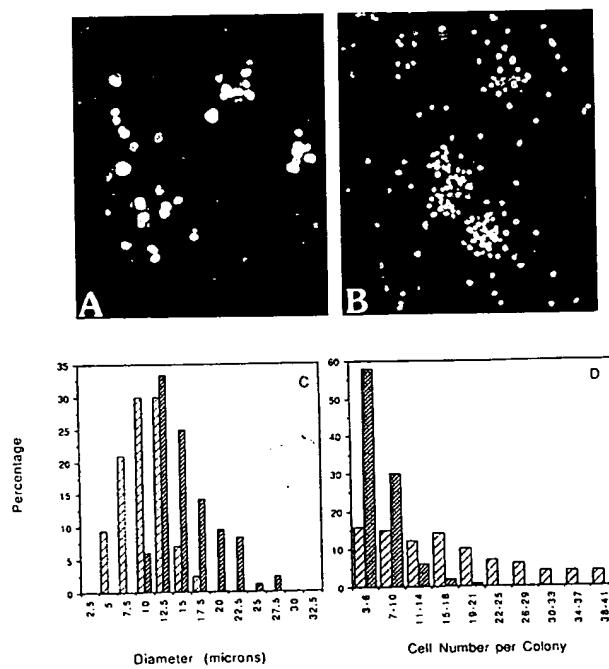
Fractions corresponding to Mono-Q ion exchange peak 2 were combined, concentrated, and applied to a gel filtration column equilibrated in 0.1% SDS (Fig 3A). Analysis of fractions by SDS-PAGE again showed a clear correlation between MGDF activity and the presence of the 25-kD protein (Fig 3B). Earlier experiments had shown that, in the absence of SDS, MGDF eluted in the void volume of the gel filtration column. The nature of this SDS-dissociable complex remains uncharacterized.

Additional purification of MGDF was performed by C4 RP-HPLC (Fig 4A). The activity, as determined by both the liquid culture human megakaryocyte assay and 32D/Mpl<sup>+</sup> survival assay, was confined to fractions 21 to 23. An SDS-PAGE analysis of two separate RP-HPLC runs is shown in Fig 4B. Fraction 21 contained a protein doublet at 31 kD, fraction 22 contained a mixture of the 25-kD and 31-kD proteins, and fraction 23 contained a highly purified 25-kD protein. Electrophoresis under reducing conditions had no effect on the apparent mobilities of the 25 and 31-kD forms of MGDF. Gel-slice elution studies of fraction 22 showed that both molecular weight forms were active in both assays and inhibitable with Mpl-X<sup>9</sup> (data not shown).

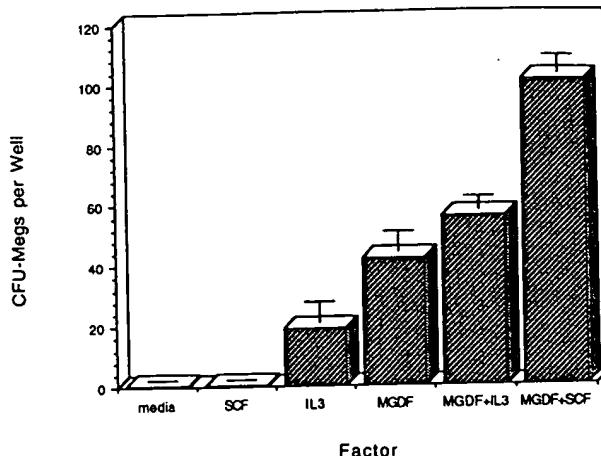
N-terminal sequence analysis of the RP-HPLC purified 25-kD protein yielded the sequence A-P-P-A-X-D-P-R-L-L-N-K-M-L-R-D-S-H-V-L-H-X-R-L-X-Q-X-P-D-I-Y, at an approximate initial yield of 19 pmol. These data extend the

MGDF concentrations of 2,500 U/mL or greater,  $269 \pm 6$  CFU-Megs were observed per 6,600 CD34<sup>+</sup> cells. Because these colonies were often confluent, this number may be an underestimate. Nonmegakaryocytic colonies were observed only occasionally; in experiments using 5,000 U/mL MGDF,  $6 \pm 3$  granulocyte/macrophage-like colonies were generated. In contrast, IL-3 at a maximally effective concentration of 2.5 ng/mL induced  $49 \pm 7$  CFU-Megs and, in addition,  $88 \pm 7$  granulocyte/macrophage-like colonies.

The cells within MGDF-induced colonies were visibly larger than those within IL-3-induced colonies. This difference is apparent in Fig 6, which illustrates megakaryocyte colonies generated with MGDF (1,000 U/mL, Fig 6A) or IL-3 (2 ng/mL; Fig 6B), both cytokines at maximally effective concentrations. Nuclei are seen as intense white spots (propidium iodide) and megakaryocytes are identified by the pale cytoplasmic halos (anti-GPIIb/IIIa and anti-GPIb). The size differences were quantitated (Fig 6C) by measuring the largest diameters of megakaryocytes fixed and stained *in situ* after 8 days of liquid culture. In MGDF-stimulated cultures,



**Fig 6. Megakaryocyte colony formation by MGDF and IL-3.** Megakaryocyte colonies were induced with (A) 1,000 U/mL MGDF or (B) 2 ng/mL recombinant human IL-3. Colonies were fixed *in situ*, stained with mouse anti-platelet glycoproteins/FITC-goat antimouse and propidium iodide, and photographed directly from the culture well. (C) The diameters of megakaryocytes generated in 8-day liquid cultures in either MGDF (1,000 U/mL, □, n = 78 cells) or IL-3 (2 ng/mL, □, n = 65 cells) were measured from video prints taken of fixed and stained cultures. A video print of a slide micrometer was used as reference. Data are presented as the percentage of the total population. Statistically significant ( $P < .001$ ) differences were seen between the groups. (D) The number of cells within megakaryocyte colonies generated in either MGDF (1,000 U/mL, □, n = 83 colonies) or IL-3 (2 ng/mL, □, n = 83 colonies) were counted from fixed and stained cultures. Data are presented as the percentage of the total population. Statistically significant ( $P < .001$ ) differences were seen between the groups.

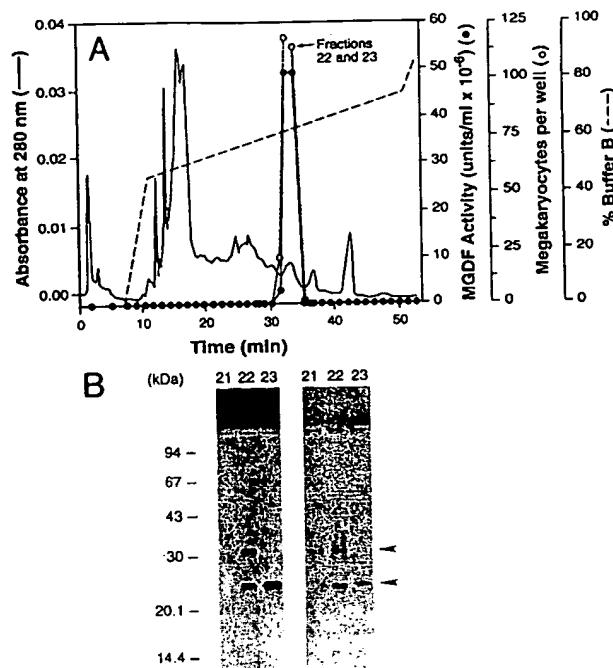


**Fig 7. MGDF is additive with IL-3 and synergistic with SCF in megakaryocyte colony formation.** MGDF at 400 U/mL, IL-3 at 1 ng/mL, and SCF at 100 ng/mL were tested alone and in combination for an ability to induce megakaryocyte colony formation from CD34<sup>+</sup> peripheral blood cells. Statistically significant ( $P < .001$ ) differences were seen between all groups and media or SCF groups and between the MGDF and MGDF + SCF groups.

the megakaryocytes ranged between 10 and 27.5  $\mu$ m in diameter with a mean value of  $15.4 \pm 0.4$  and a median value of 15. In IL-3-stimulated cultures, the megakaryocytes ranged between 5 and 17.5  $\mu$ m in diameter with a mean value of  $10.3 \pm 0.5$  and a median value of 10. The probability that the two populations were statistically equivalent was  $P < .001$ .

The number of cells with MGDF-induced colonies were fewer than those within IL-3-induced colonies (Fig 6D). In MGDF-induced cultures, from 3 to 20 cells were found per colony with a mean value of  $6.6 \pm 0.4$  and a median value of 6. The distribution pattern was compact with 78% of the colonies containing 8 cells or less. Only 1 of 83 colonies showed evidence of more than 4 divisions (20 cells per colony). In IL-3-induced cultures, from 3 to 41 cells were found per colony with a mean value of  $17 \pm 1$  and a median value of 16. The distribution varied widely with 10% of the colonies containing 3 to 4 cells and 11% of the colonies containing 33 to 64 cells. The probability that the two populations were statistically equivalent was  $P < .001$ .

**MGDF is synergistic with SCF but not IL-3 in megakaryocyte colony formation.** Purified MGDF was tested in combination with IL-3 or SCF in megakaryocyte colony-formation assays. Five independent combination studies were performed with both submaximal and maximal concentrations of MGDF and IL-3. SCF, which had no effect in the assay at any concentration tested, was always used at 100 ng/mL. In Fig 7, the results using submaximal concentrations of MGDF (400 U/mL) and IL-3 (1 ng/mL) are shown. This combination resulted in an additive response in which  $42 \pm 7$  CFU-Megs were observed with MGDF alone,  $19 \pm 7$  with IL-3 alone, and  $56 \pm 4$  with the combination. In contrast, the combination of MGDF and SCF resulted in a synergistic response such that  $101 \pm 6$  CFU-Megs were observed. This data set is statistically different from the data using MGDF



**Fig 4.** RP-HPLC purification and SDS-PAGE analysis of MGDF. The active fractions from gel filtration were applied to a C<sub>4</sub> reverse-phase microbore column as described in Materials and Methods. (A) A representative profile is shown. MGDF levels were determined with the 32D/Mpl+ cell line (●) and also by megakaryocyte development (○). (B) Aliquots of fractions 21, 22, and 23 from two independent C<sub>4</sub> runs were analyzed with 14% nonreducing SDS-PAGE and silver stained. Arrows indicate positions of MGDF forms.

sequence to 31 cycles from the 21 cycles reported earlier. A computer-based search of available data bases indicated that this sequence was novel.<sup>9</sup> Two additional sequences, at initial yields of 2 pmol, were also detected in this sample. These two sequences were identified as canine IgG light and heavy chains and corresponded to a high molecular weight contaminant observed in these fractions by SDS-PAGE (Fig 4B). These results indicate that the 25-kD protein comprised 90% of the detectable protein in the sample. N-terminal sequence analysis of the fraction containing the 31-kD proteins yielded a sequence (5 pmol) identical to that of the 25-kD protein, although the amount of sample limited this analysis to 21 cycles.<sup>9</sup> Sequences corresponding to canine Ig heavy (3 pmol) and light chains (3 pmol) were also detected in this sample.

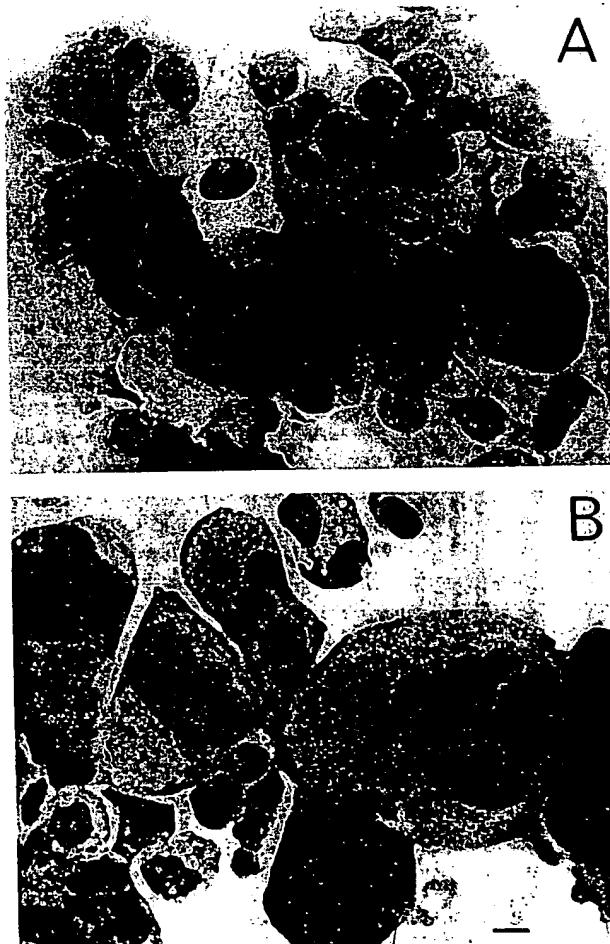
#### Biologic Properties of MGDF *In Vitro*

**Liquid culture.** Purified 25-kD and 31-kD MGDF (from C4-RP-HPLC fractions 23 and 21, respectively) both generated megakaryoblasts and megakaryocytes in liquid culture (Fig 5A and B). All four stages of mature megakaryocytes were observed in these cultures (J. Nichol, unpublished observations). Equivalent responses were observed whether the two forms were used individually or together. The percentage of megakaryocytes observed in these liquid cultures ranged from 20% to 100% (mean of 66%  $\pm$  10%; n = 6

determinations). The remaining cells were undefined blast cells, small mononuclear cells, macrophages, or occasionally basophils. Benzidine-positive cells were not observed in MGDF-stimulated cultures, even with IL-3 added as cofactor (data not shown).

**MGDF is a Meg-CSF.** The following describes data from 5 independent experiments that used an MGDF preparation equivalent to the active pool shown in Fig 3, fractions 43 to 46. Based on the silver staining pattern of the analytical gels, the purity was estimated as 30%. The 25-kD and 31-kD proteins were present at an estimated ratio of 4:1. The dominant protein contaminants were identified as canine Ig heavy and light chains.

The abilities of MGDF and IL-3 to specifically induce megakaryocyte colonies from CD34<sup>+</sup> cells were compared. The response to MGDF was linear and megakaryocyte specific from 250 to 2,500 U/mL. At maximally effective



**Fig 5.** Stimulation of megakaryocyte development in liquid culture by 25-kD and 31-kD MGDF species. Forty thousand CD34<sup>+</sup> peripheral blood cells were incubated for 9 days in either (A) 10,000 U/mL MGDF 25 kD, or (B) 5,000 U/mL MGDF 31 kD in 150  $\mu$ L in flat-bottomed, 96-well culture plates for 9 days. Cells were harvested, centrifuged, and stained by a modified Wright-Giemsa method. Slides were photographed with the Microphot-FXA microphotography system. Reference bar = 10  $\mu$ m.

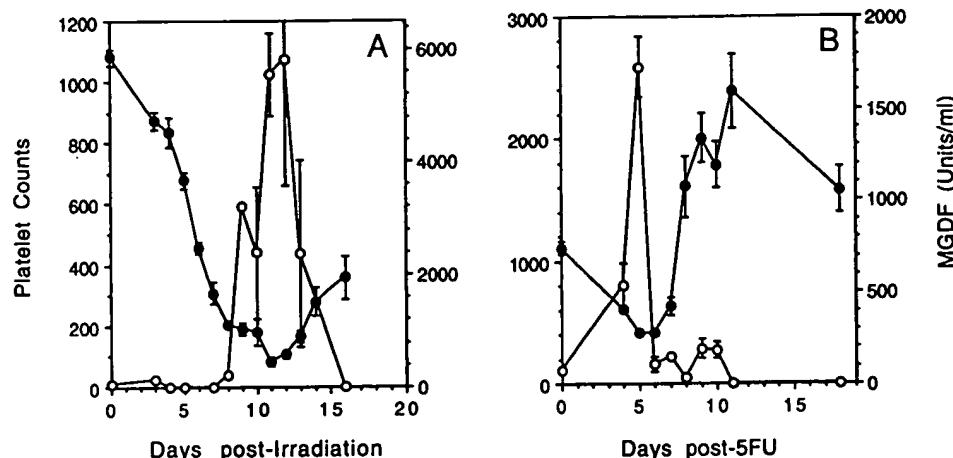


Fig. 8. MGDF is elaborated during periods of thrombocytopenia in rodent models. Mice were rendered thrombocytopenic and monitored for platelet counts ( $\times 1,000/\mu\text{L}$ , ●) and serum MGDF levels (U/mL, ○) over time. MGDF levels were determined on the 32D/Mpl<sup>+</sup> cell line. (A) Balb/c mice ( $n = 10$ ) were irradiated with 500 rad on day 0. (B) DBA mice ( $n = 5$ ) were injected with 5-FU on day 0.

alone at  $P < .001$ . These same phenomena of MGDF/IL-3 additive effects and MGDF/SCF synergistic effects were observed when maximally effective concentrations of MGDF (1,000 U/mL) and IL-3 (50 ng/mL) were used (data not shown).

*Serum levels of MGDF in in vivo models of reversible thrombocytopenia.* Experiments presented above that measured canine plasma MGDF levels after irradiation showed an apparent inverse relationship between activity and platelet counts. Because this model of marrow aplasia was lethal, it was not possible to also evaluate MGDF levels during platelet recovery. Therefore, serum MGDF levels were determined against platelet counts in three murine models of reversible thrombocytopenia: sublethal irradiation, 5-FU (Fig 8A and B, respectively), and immune-mediated thrombocytopenia (Table 1). In mice treated with sublethal irradiation, serum MGDF was detectable by 8 days when the platelet count had decreased to 20% of normal, peaked in concentration to  $5,806 \pm 2,214$  U/mL at the time of the platelet nadir of  $80,000/\mu\text{L}$  (11 to 12 days), and was no longer detectable by 16 days as the platelets returned towards baseline values. In mice injected with a single dose of 5-FU, serum MGDF was detectable by 4 days when the platelets were 60% of normal, peaked in concentration by 5 days to  $1,727 \pm 167$  U/mL as the platelets decreased to a nadir of  $400,000/\mu\text{L}$ , and then decreased rapidly in concentration to undetectable levels after 12 days. In mice injected with a single dose of rabbit antimouse platelet antisera, serum MGDF was

observed only in a sharp peak of  $4,266 \pm 870$  U/mL 24 hours after treatment. Animals receiving normal rabbit sera did not produce MGDF. In all three models of reversible thrombocytopenia, serum MGDF levels were greatest during thrombocytopenic periods and generally undetectable when platelet counts were at normal levels.

## DISCUSSION

MGDF, defined as an activity in aplastic canine plasma that induces in vitro human megakaryopoiesis,<sup>11-14</sup> has been cloned, expressed, and characterized as a ligand for Mpl.<sup>9</sup> However, an in-depth presentation of the purification of this important factor to homogeneity and a biologic characterization of highly purified, serum-derived MGDF have not yet been presented. Two molecular weight species of MGDF sharing the same N-terminal amino acid sequence were isolated. Multiple forms of this cytokine with similar molecular weights were also purified from thrombocytopenic porcine<sup>4</sup> and sheep<sup>8</sup> plasma, whereas a single 19-kD species was isolated from irradiated rat plasma.<sup>7</sup> Both the 25- and 31-kD canine MGDF forms are active in the 32D/Mpl<sup>+</sup> and human megakaryocyte assays at estimated concentrations of 0.5 to 15 pg/mL, based on yields from N-terminal sequence analysis, and behave additively when tested in combination. The basis for the molecular weight differences between serum-derived species is not known at this time. Because the supply of the purified canine protein is limited, the identification of glycosylation, proteolytic, or splicing events that led to the

Table 1. Serum MGDF Levels in Mice During Immune-Mediated Thrombocytopenia

Assay	Time								
	Day 0	2 h	8 h	Day 1	Day 2	Day 4	Day 6	Day 8	
APS	Platelets	$1,103 \pm 69$	$122 \pm 34$	$45 \pm 11$	$131 \pm 36$	$312 \pm 22$	$1,435 \pm 38$	$1,672 \pm 36$	$1,372 \pm 62$
	MGDF	0	0	0	$4,266 \pm 870$	0	0	0	0
NRS	Platelets	$1,117 \pm 18$	$985 \pm 28$	$1,023 \pm 20$	$1,168 \pm 40$	$1,074 \pm 46$	$1,156 \pm 88$	$1,093 \pm 88$	$1,208 \pm 4$
	MGDF	0	0	0	0	0	0	0	0

DBA mice were injected intraperitoneally on day 0 with a single dose of APS or NRS. At the indicated times, platelet counts ( $\times 10^{-3}/\mu\text{L}$ ) and serum MGDF (U/mL) were determined. MGDF levels were measured with the 32D/Mpl<sup>+</sup> cell line. Data are expressed as the mean  $\pm$  SEM ( $n = 5$  animals).

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multiple forms must await the development of MGDF site-specific immunologic or nucleic acid probes.

Serum/plasma levels of MGDF were generally inversely related to the circulating platelet count in experimental models of marrow ablation or immune-mediated thrombocytopenia. In models of canine or murine irradiation, MGDF was first detected when platelets were 20% to 30% of normal and continued to increase as the platelet count decreased. In the murine model of reversible thrombocytopenia, MGDF levels started to return towards baseline just as the platelet count started recovering. The pattern seen after chemotherapy with 5-FU was similar but not identical. MGDF was first detected at a platelet count of 600,000/ $\mu$ L and peaked at the nadir of 400,000/ $\mu$ L, which, in the irradiated model, were not platelet counts associated with serum MGDF. Additionally, MGDF levels at day 6 post-5-FU were very low, despite the minimal platelet count. This report does not offer data to explain the differences seen in the two models of marrow aplasia, but mouse strain differences, differential damage to MGDF-producing organs, or differences in the classes of surviving progenitor cell populations drawing on the factor for their development may account in part for the apparent discrepancies. The pattern of serum MGDF observed in a model of immune-mediated thrombocytopenia was also slightly different. MGDF levels peaked at 24 hours instead of precisely with the platelet nadir of 45,000/ $\mu$ L occurring at 8 hours. This observation, also reported by Wendlung et al,<sup>26</sup> could indicate a minimal time requirement for MGDF induction/production and accumulation. That MGDF is not observed after 24 hours could indicate rapid use of the cytokine. In fact, platelet levels return to control values within 2 to 3 days after the peak of MGDF accumulation.

The factor purified from aplastic canine plasma behaved as a classic Meg-CSF that at maximal concentrations was approximately 5 times more effective in generating human megakaryocyte colonies than recombinant human IL-3. MGDF-induced colonies contained fewer cells of larger size than did IL-3-induced colonies, suggesting either that the MGDF-target cell is a more mature progenitor cell than the IL-3 target cell or that the cytokines act on the same population in different ways. MGDF may encourage more endomitotic divisions (at the expense of mitotic divisions) than IL-3, at least over the dose ranges tested. Like other colony-stimulating factors,<sup>27,28</sup> MGDF was synergistic with SCF on the generation of megakaryocyte colonies. In contrast, MGDF in combination with IL-3 had an additive effect. This finding is in apparent contrast with data derived using the murine Mpl ligand with unfractionated, murine bone marrow cultures.<sup>6</sup> The different results might reflect the species, the degree to which the different target populations are enriched for progenitors, or a difference in the progenitor cell populations within peripheral blood compared with marrow. Plasma-derived canine MGDF shows relative, although not absolute, lineage specificity on human progenitors. A small number of myeloid colonies were observed with supramaximal factor concentrations. In 8-day liquid cultures of CD34-selected cells, MGDF generated cell populations that were predominantly, although not always exclusively, megakaryocytic. With increased culture time the percentage of mega-

karyocytes increased, presumably as unidentifiable megakaryocyte precursors reached maturity (J. Nichol, unpublished data). Erythroid cell development was never observed with MGDF, even in combination with IL-3. These results using purified canine MGDF derived directly from aplastic plasma on human cells are generally in agreement with studies performed with recombinant human<sup>9,10</sup> or murine<sup>3</sup> factors despite the species mismatches. In current studies in which recombinant human MGDF is being administered to normal dogs, platelet counts are increased fourfold to fivefold (Farese et al, manuscript in preparation). In summary, MGDF derived from aplastic canine plasma is a glycosylated cytokine existing in at least two related forms that behaves as a lineage-restricted, colony-stimulating factor of the same class as erythropoietin, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and IL-5, cytokines that direct lineage-committed progenitors through their final mitotic divisions and into terminal differentiation.<sup>29,30</sup>

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